Antibiotic Efficacy in Treating Variant *Pseudomonas aeruginosa* and *Staphylococcus aureus* Biofilms

Undergraduate Research Thesis

By

Anthony Li
The Ohio State University
College of Pharmacy

Principal Investigator
Paul Stoodley, Ph.D.
Department of Microbial Infection and Immunity
College of Medicine

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Abstract

This project is a branch off of another project that involves finding transcriptional changes expressed in variant colonies within *Pseudomonas aeruginosa* biofilms. Preliminary *in vitro* studies have shown that in the presence of a tobramycin bead, many different phenotypic colonies, dubbed variants, appeared within the biofilm. *P. aeruginosa* is normally susceptible towards tobramycin, but these variant colonies are able to survive the effects of the antibiotic whether through tolerance, resistance, or by some other mechanism. In a separate study testing antibiotic bead combinations with *Staphylococcus aureus*, a similar phenomenon was occurring with gentamicin. Although it is uncertain that these variant colonies are similar to *P. aeruginosa*, for this project both types of colonies fall under the umbrella term variant. Being able to understand these variant colonies of bacteria can greatly improve the approach when treating bacterial infections, especially when it comes to recurring infections.

This study investigates antibiotic efficacy against planktonic vs biofilm bacteria *in vitro*, using eight different antibiotics: carbenicillin, ciprofloxacin, colistin, gentamicin, meropenem, rifampicin, tobramycin, and vancomycin. The main aim is to see which antibiotic can best clear the biofilm, which antibiotics correlate to the appearance of variant colonies, and which antibiotics can eradicate variant colonies. Biofilms are resilient toward chemical and physical challenges due to the secretion of extracellular polymeric substance and high cellular density. This makes biofilm infections difficult to treat. Some examples of biofilm infections are osteomyelitis and cystic fibrosis pneumonia most commonly caused by *S. aureus* and *P. aeruginosa* respectively. This study uses bioluminescent bacteria, *S. aureus* SAP231 and *P. aeruginosa* Xen41 as the test subjects for antibiotic efficacy. There are four major components to this study: first, the minimum inhibitory concentration of planktonic cells against antibiotics were found. Second, the antibiotic delivery mechanism was tested to see if mode of antibiotic delivery has an effect on biofilm clearance. Third, antibiotics and combinations of antibiotics are tested against biofilms. Lastly, the variant colonies are quantified to see which antibiotics correlate to variant colony formation. The results show that biofilm infections are more difficult to treat than planktonic bacteria, and the use of multi-combinatory antibiotics are effective at treating biofilm. A combination of antibiotic with vancomycin was very effective in reducing biofilm and variant colonies in *S. aureus*. A combination of antibiotic with ciprofloxacin was very effective in reducing biofilm and variant colonies in *P. aeruginosa*.

Acknowledgements

I would like to thank Dr. Stoodley for giving me the opportunity to be a part of the lab and exposing myself to the field of infectious disease. I was able to learn much about the process of research and how to conduct my own experiments. Dr. Stoodley has guided me through the process, giving me great ideas on how to approach my experiments. He was always willing to edit my presentations and paper always providing positive feedback to improve my skills. I am very grateful for the opportunity he has given me.

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Introduction

Bacterial biofilms have been correlated with chronic and persistent infections due to the difficulty in treating the infection completely with antimicrobial agents.[1] Bacterial biofilms form when planktonic, free swimming, bacteria attach to a surface and encase themselves in an extracellular polymeric substance (EPS).[2] The extracellular matrix provides the biofilm with a whole host of benefits including resilience towards chemical and physical challenges. Some protective mechanisms include poor penetration of antibiotics through the EPS, slow growth of bacteria with the efficacy of many antibiotics depending on active growth of bacteria, and the formation of persisters, which are a subpopulation of bacteria that are neither able to grow nor die.[3] Another mechanism is the gaining of antibiotic resistance. Frequent mutations in a high density cell population combined with horizontal gene transfer, has led biofilms to contribute to the rise of antibiotic resistance.[2] All of these tolerant/resistant mechanisms make treating biofilms more difficult compared to planktonic infections, where planktonic infections respond well to antibiotic treatments.[2] Some examples of biofilm related infections include osteomyelitis, cystic fibrosis pneumonia, and medical device implant-related infections. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are some of the most common biofilm forming bacteria that cause infection, *P. aeruginosa* being most common in cystic fibrosis pneumonia and *S. aureus* being the most common in osteomyelitis.[1] Combined with the rise of antimicrobial resistances and the resilience of biofilms, biofilm-related infections are extremely difficult to treat.

A treatment option for osteomyelitis and other orthopedic related procedures is the use of calcium sulfate ($CaSO_4$) beads impregnated with antibiotics.[5] These beads are surgically implanted in the wound site allowing high localized elution of antibiotics, which is important because biofilms have antibiotic tolerant properties.[3] The advantage of using these beads is having the capacity to use higher concentrations of antibiotics to treat the infection while lowering instances of antibiotic induced toxicity because of the localized elution.[4] When it comes orthopedics, gentamicin loaded bone cement is used during surgeries.[17] In an *in vitro* study testing $CaSO_4$ beads on *S. aureus* biofilms, a gentamicin loaded bead was placed in the center of the biofilm. After 8 days of incubation, the use of a gentamicin bead lead to the appearance of variant colonies within the biofilm (Figure 1). A similar event was occurring with *P. aeruginosa*, in which a gentamicin loaded beads also led to the appearance of variant colonies after 4 days of incubation (Figure 2). These findings were surprising because *S. aureus* and *P. aeruginosa* are normally susceptible to gentamicin[6,7], but over the course of time, variant colonies appeared in what seemed to be a dead biofilm. This phenomenon could help explain persistent and chronic infections based on the emergence of antibiotic variant colonies within a biofilm.

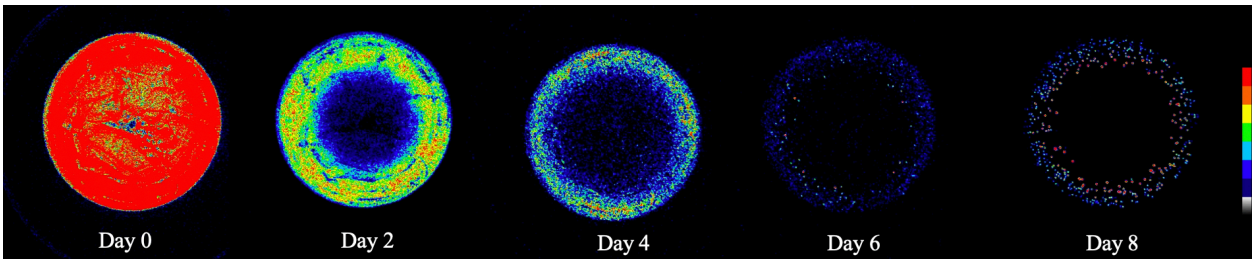


Figure 1: IVIS image shows the effects of a gentamicin $CaSO_4$ bead against *S. aureus* SAP231 biofilm. As time progresses, the zone of killing increases. On day 8, variant colonies appeared within the zone of killing within the biofilm. Red on the IVIS indicates metabolically active bacteria while blue indicates less activity. Red dots on the IVIS are identified at variant bacteria.

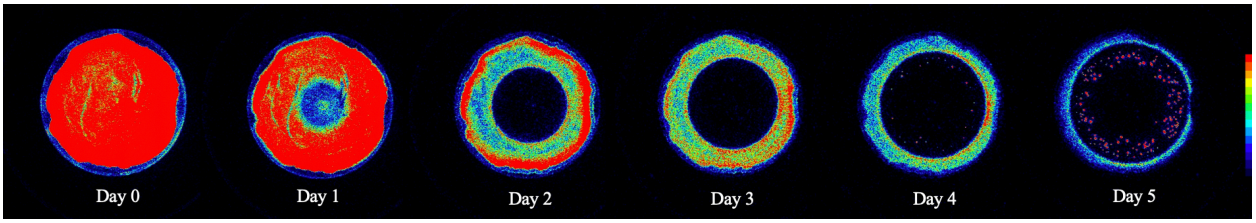


Figure 2: IVIS image shows the effect of a gentamicin $CaSO_4$ bead against *P. aeruginosa* Xen41 biofilm. As time progresses, the zone of killing increases. On day 4, variant colonies appeared within the zone of killing within the biofilm. Red on the IVIS indicates metabolically active bacteria while blue indicates less activity. Red dots on the IVIS are identified at variant bacteria.

Variant bacteria, in the context of the colonies seen on the antibiotic loaded plate, is an umbrella term that encompasses tolerant, resistant, and other variants of bacteria. Replica plating is a technique used to differentiate the variant colonies. In replica plating, the original plate containing the colonies is stamped onto a velveteen cloth. Then new agar media containing antibiotic and no antibiotic are stamped onto the cloth. This transfers the bacteria from one plate to another keeping the same pattern. Variant colonies are classified into 3 categories tolerant, resistant, and viable but not culturable (VBNC) (Figure 3). Tolerant being defined as when the bacteria have the ability to survive in the presence of antibiotics, but will revert back to being susceptible once re-cultured in an antibiotic environment. Resistant is when the bacteria will remain being resistant to an antibiotic once removed from and re-cultured onto an antibiotic environment. VBNC colonies appear in the initial plate, but are unable to be re-cultured onto new media with and without antibiotics present.

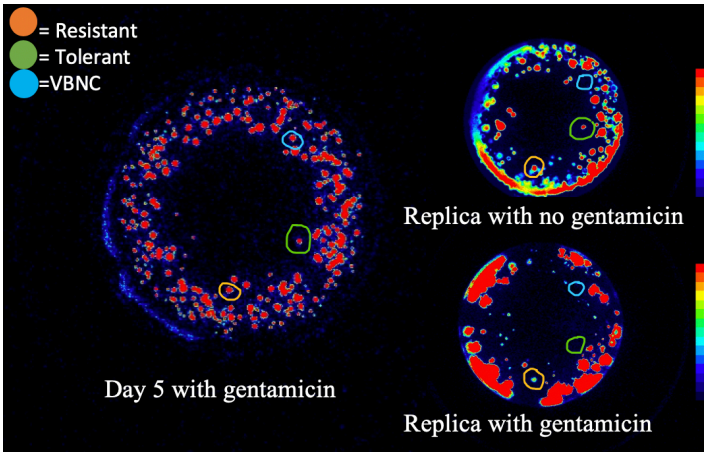


Figure 3: Variant colonies appear on IVIS images of Xen41 grown with a gentamicin bead. Replica plating was done to differentiate the different types of variants. The original plate was replica plated onto a new plate containing no gentamicin and a plate with gentamicin. Resistant, tolerant, and VBNC colonies can be seen based on the appearance of the colony on the original plate compared to the replica plates.

To investigate the antibiotic variant phenomenon, different antibiotic classes were tested against bioluminescent strains of *P. aeruginosa* and *S. aureus*, looking for which antibiotics will be most effective in treating the biofilm and preventing the appearance of antibiotic variant bacteria. *P. aeruginosa* and *S. aureus* were chosen not only because they are common bacterial infections, but they encompass both gram-positive and gram-negative bacteria. The classification of the bacteria's Gram stain can correlate to which classes of antibiotics the bacteria will be susceptible to. The hypothesis here is that other antibiotics can lead to the development of variant colonies and treating the biofilm with multidrug combinations will be most effective at eradicating the biofilm and reduce variant numbers. The goal of this project is to find which antibiotic(s) will be most effective in treating biofilms, which antibiotics correlate to the appearance of variant colonies, and which antibiotics can eradicate variant colonies.

Materials and Methods

The overview of the experiments can be broken down into 4 parts. First, the minimum inhibitory concentration (MIC) was found for each antibiotic against *S. aureus* and *P. aeruginosa* to see the efficacious dose of the antibiotics, which will be used as a comparison model for planktonic v. biofilm bacteria. Second, the antibiotic delivery system is tested to see if the mode of delivery of $CaSO_4$ and paper disc will produce significantly different results. Third, single and multidrug combinations are tested to see which antibiotic(s) will be most effective in clearing the biofilm. Lastly, variant colony formation will be measured to see which antibiotic(s) is most effective in clearing or reducing the number of variant colonies.

Materials 1: Bacterial Strains and Imaging

***Pseudomonas aeruginosa* Xen41 (Xen41)** is a bioluminescent strain of *P. aeruginosa* derived from parental strain PAO1 with an inserted lux operon from *Photorhabdus luminescens*.[8]

***Staphylococcus aureus* SAP231 (SAP231)** is a bioluminescent strain of *S. aureus* derived from USA 300 MRSA with an inserted lux operon from *Photorhabdus luminescens.*[8,11]

***In vivo* imaging system** (IVIS) allows the imaging of bioluminescence. Bacterial metabolic activity can be measured with IVIS.

ImageJ- ImageJ is a program that allows measurements to be made on the IVIS images.[14]

Materials 2: Antibiotics

Carbenicillin (Carb) (GoldBio) is a broad-spectrum semisynthetic beta-lactam drug that interferes with cell wall synthesis by preventing the cross linking of peptidoglycan.[9]

Ciprofloxacin (Cipro) (Sigma-Aldrich) is a broad-spectrum fluoroquinolone that inhibits DNA gyrase and topoisomerase IV, which is required for DNA replication. [9]

Colistin Sulfate (Colistin) (GoldBio) is a type of polymyxin that disrupts and change cellular membrane permeability due to polycationic properties of colistin being both lipophilic and hydrophobic. [9]

Gentamicin Sulfate (G) (GoldBio) is a broad-spectrum aminoglycoside that irreversibly bind to 30S ribosomal subunit interfering with protein synthesis. [9]

Meropenem (M) (GoldBio) is a broad-spectrum carbapenem that inhibits cell wall synthesis by interfering with the cross linking of peptidoglycan. [9]

Rifampicin (R) (GoldBio) is a broad-spectrum semisynthetic antibiotic that inhibits DNA-dependent RNA polymerase, thus inhibiting RNA synthesis. [9]

Tobramycin Sulfate (T) (GoldBio) is an aminoglycoside that irreversibly binds to the 30S ribosomal subunit interfering with protein synthesis. [9]

Vancomycin Hydrochloride (V) (GoldBio) inhibits cell-wall synthesis by preventing N-acetylmuramic acid and N-acetylglucosamine from binding together by binding to D-Ala-D-Ala in the peptidoglycan matrix. [9]

Materials 3: Compounding of Antibiotics

Calcium sulfate beads were made with desired amount of antibiotics with calcium sulfate hemihydrate (Sigma-Aldrich) (20g) and distilled water (6mL), casting the paste in 4.2mm bead mold and set to dry for 24hrs. The amounts can be scaled depending on how many beads are requir

Paper discs (6.35mm) (Thermo-Scientific) were made using antibiotic solution. Antibiotic solution was made using desired amount of antibiotic with distilled water (1mL). The amount of solution pipetted onto the disc depended on the desired concentration.

Materials 4: Media and Biofilm Preparation

Lysogeny broth (LB) and **LB agar (LBA)** (Fisher Scientific) were used as the nutrient source to culture *P. aeruginosa*. LB was made by suspending LB powder (12.5g) with distilled water (500mL) (dH_2O) and autoclaving to sterilize the media. LBA was made my suspending LB powder (12.5g) with dH_2O (500mL) and agar (7.5g). The solution was autoclaved and pipetted (20mL) into sterile 100mm*15mm petri dishes (Fisher Scientific).

Brain Heart Infusion broth (BHI) and **BHI agar (BHIA)** (Beckton, Dickinson and Company) were used as the nutrient source to culture *S. aureus*. BHI was made by suspending BHI powder (18.5g) with distilled water (500mL) (dH_2O) and autoclaving. BHIA was made by suspending BHI powder (18.5g) with dH_2O (500mL) and agar (7.5g) (Fisher Scientific). The solution was autoclaved and pipetted (20mL) into sterile 100mm*15mm petri dishes.

Bacterial biofilms were formed on agar plates. The bacteria were grown in broth culture overnight in a shaking incubator at 37°C. The bacterial culture was then diluted to an optical density (OD_{600}) of 0.10X ($1*10^6$ cells/mL). The diluted culture (100µL) was then spread onto an agar plate using a lawn spreader. The agar plate was incubated at 37°C overnight to allow the biofilm to form.

Method 1: Minimum Inhibitory Concentration of Planktonic Bacteria (n=3)

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of a drug that will prevent the visible growth of bacteria.[10] This assay was performed to get reference values for the efficacy of antibiotics against SAP231 and Xen41 in planktonic form. This data can be used to compare planktonic v. biofilm bacteria. The experiment used a 96-well plate with each row containing a different antibiotic and each column containing a different concentration. Antibiotic solution was diluted down into each well (Table 1) and diluted overnight culture was added, so that each well contained a total concentration of $1*10^6$ cells/mL.[12] The blanks only contained broth and no culture. The 96-well plate was incubated at 37°C for 24hrs. The OD_{600} was taken via microplate reader setting the well with just the broth as a blank. The MIC can be determined based on the concentration v. OD_{600} graph. The values for the MIC were determined based by the bottom plateau of the graph, where there were no changed in optical density indicating no growth.

Table 1: MIC 96-Well Plate Set-Up

Well #	1	2	3	4	5	6	7	8	9	10	11
Antibiotic (µg/mL)	64	32	16	8	4	2	1	0.5	0.25	0	Blank

Method 2: Antibiotic Elution of $CaSO_4$ and Paper discs (n=3)

Throughout the course of this study, different modes of antibiotic delivery were used in the eradication of biofilm. This experiment was done to account for the similarities and differences between the antibiotic delivery materials. Two modes of antibiotic delivery were used, $CaSO_4$ beads and paper discs. In this experiment, $CaSO_4$ beads and paper discs impregnated with gentamicin (1mg) are tested against Xen41. A control group was set up with $CaSO_4$ bead and paper disc with no antibiotic added. The antibiotic containing vessels were then placed in the center of an agar plate with pre-grown Xen41. Xen41 was incubated for 5 days at 37°C, taking IVIS images every 24 hrs. The zones of killing, zones of variance, and zones of biofilm were compared between the different treatment materials (Figure 4). An ANOVA test was done to test for significance.[13] ANOVA is an analysis of variance that test differences of means from multiple treatment groups.

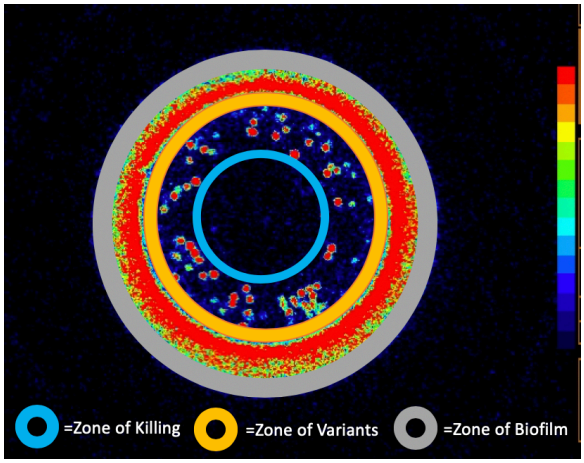


Figure 4: In the presence of gentamicin, Xen41 produced three distinct zones. These zones are classified as the zone of killing, where no bacteria were able to grow, the zone of variants, where variant colonies grew, and the zone of biofilm, where the biofilm remained intact. Data for experiments 2 and 3 use the radius of the zone to calculate the area of each zone.

Method 3: Efficacy of Single and Combinatory Antibiotic(s) against Biofilm (n=3)

A preliminary experiment was done to see if the diffusion of the antibiotic is being hindered by the agar media. To test this, one antibiotic bead (1mg) was placed onto the center of the agar media. BHIA was used with SAP231 and LBA was used with Xen41. The agar media was incubated for 5 days at 37°C, to let the antibiotic diffuse throughout the plate. Planktonic Xen41 and SAP231 was spread onto the agar plates and incubated for 24hrs. IVIS images were taken to see if there was growth of bacteria on the plate.

This main experiment looked at the efficacy of antibiotics against Xen41 and SAP231 biofilms. Xen41 and SAP231 were first tested against carbenicillin (Carb) (1mg), ciprofloxacin (Cipro) (1mg), colistin (Colis) (1mg), gentamicin (G) (1mg), meropenem (M) (1mg), rifampicin (R) (1mg for Xen41, 2mg for SAP231), tobramycin (T) (1mg), and vancomycin (V) (1mg Xen41, 4mg SAP231). Combinatory antibiotics were tested to see if efficacy improves when treating a biofilm. Xen41 was tested against T+Carb, T+Colis, T+G, and T+M (1mg of each antibiotic). SAP231 was tested against V+G, V+R, V+T, V+R+M, and V+R+T (4mg vancomycin, 1mg gentamicin and tobramycin, 2mg meropenem and rifampicin). The amount of antibiotic used were based on antibiotic mixing guidelines for $CaSO_4$ beads, if there wasn't a listed amount for a specific antibiotic, 1mg was assigned. The antibiotics were tested against pre-grown biofilm using any of the antibiotic delivery methods. The plates were incubated at 37°C taking IVIS images every 24hrs. Xen41 was incubated for 5 days while SAP231 was incubated for 9 days. The percent coverage of the zone of killing, zone of variants, and zone of biofilm was found by using ImageJ analysis (Figure 4). The percent zone coverage can be calculated by measuring the area of each zones comparing it to the total area of the plate. The zones of killing, zones of variants, and zones of biofilm were compared on day 5 for Xen41 and day 9 for SAP231.

$$Area\ of\ zone = \pi r^2$$

$$Biofilm\ Eradication = \%\ Zone\ of\ Killing + \%\ Zone\ of\ Variants$$

To determine the efficacy of the antibiotic and combinations, the zone of killing and zone of variants were combined. The zone of variants was included in the data analysis because the antibiotic was able to clear the biofilm from this area. Looking at the cellular density of the zone of variants and the zone of biofilm, there is less bacteria in the zone of variants compared to the

complete coverage of bacteria in the zone of biofilm. (To see percent coverage of each zone refer to Appendix) A Dunnett test was done to compare the efficacy of other antibiotic against gentamicin. A Dunnett test is a statistical comparison of multiple treatment groups against a control. Gentamicin is used as the control because it is used as a standard treatment in orthopedics. A Turkey test was then used to compare the different antibiotics to each other. A Turkey test is a statistical comparison for three or more variables. Similar to the Dunnett test, a Turkey test compared the treatment groups to all treatment groups, instead of just one control. From the Turkey test the treatment group that performed the best in eradicating the biofilm can be determined.

Method 4: Quantifying Variant Colonies via Colony Density (n=3)

The variant colonies were quantified to see if certain classes of antibiotics had favorable conditions for variant bacteria appearance and which antibiotic had the fewer variant colonies. The antibiotics and combinations of antibiotics used is listed in Experiment 3. To quantify the number of variant colonies, IVIS images was used. The color scale of the IVIS image indicates metabolic activity with the color red indicating high activity and blue indicating low activity. A red dot on the IVIS image indicates a metabolically active variant colony. The variant colonies accounted for are tolerant, resistant, and VBNC phenotype. The total number of variant colonies were found by dividing the zone of variants on the IVIS image into 4 quadrants (Figure 5). The number of colonies were counted in one quadrant and multiplied by 4 to get the total number. The area within the zone of variants was also found by multiplying the percentage of the area in the IVIS image by the total area of the actual plate. The number of variant colonies per area was compared between each susceptible antibiotics and combinations.

$$\#\ of\ variant\ colonies = \#\ of\ colonies\ in\ a\ quadrant * 4$$

$$Area\ zone\ of\ variance\ = \%\ area\ encompass\ IVIS * area\ of\ the\ plate\ (78.53cm^2)$$

$$Variant\ Colonies\ per\ area = \frac{\#\ of\ variant\ colonies}{area\ zone\ of\ variance}$$

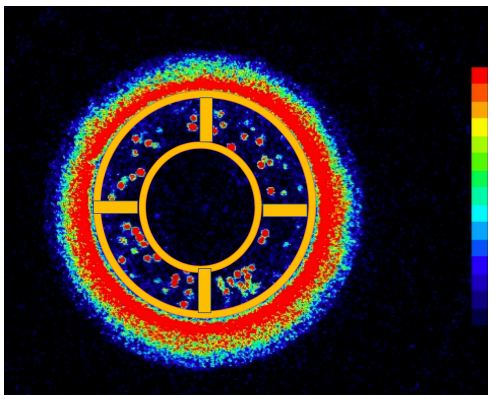
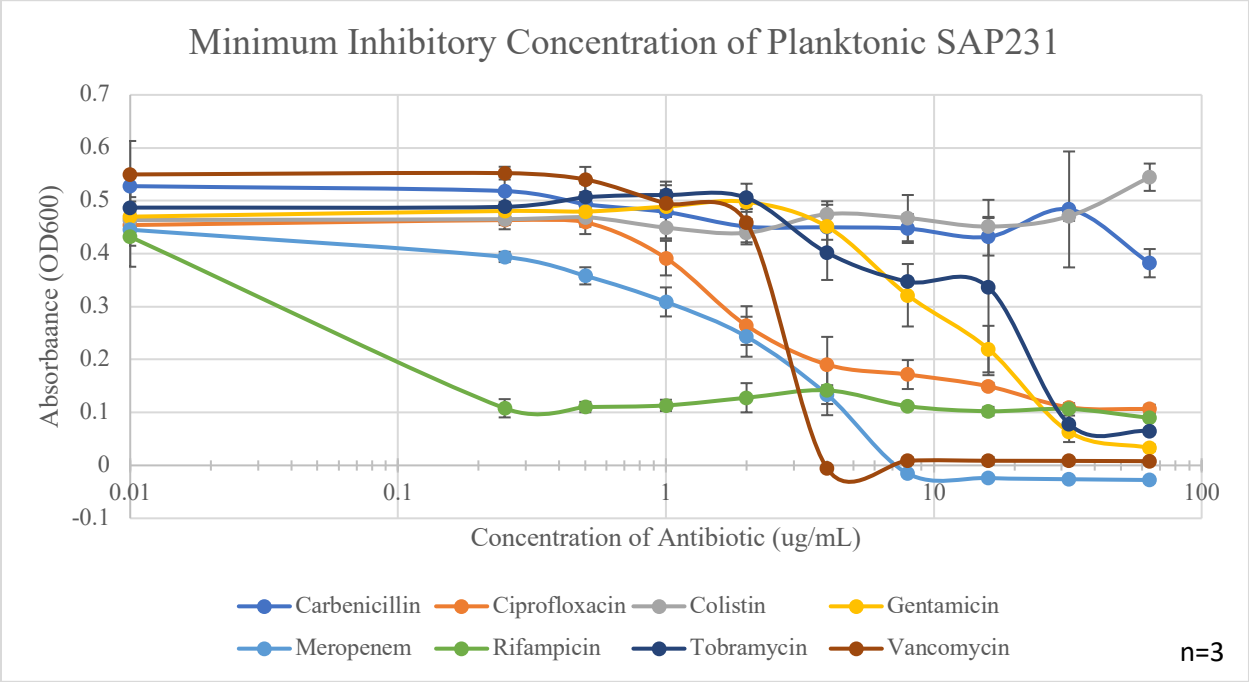


Figure 5: To quantify the variant colonies within the zone of variants the zone is divided into 4 sections. The number of colonies were counted and multiplied by 4 for the total count. The area of the zone was found in respect to the actual size of the agar plate to get the colony density.

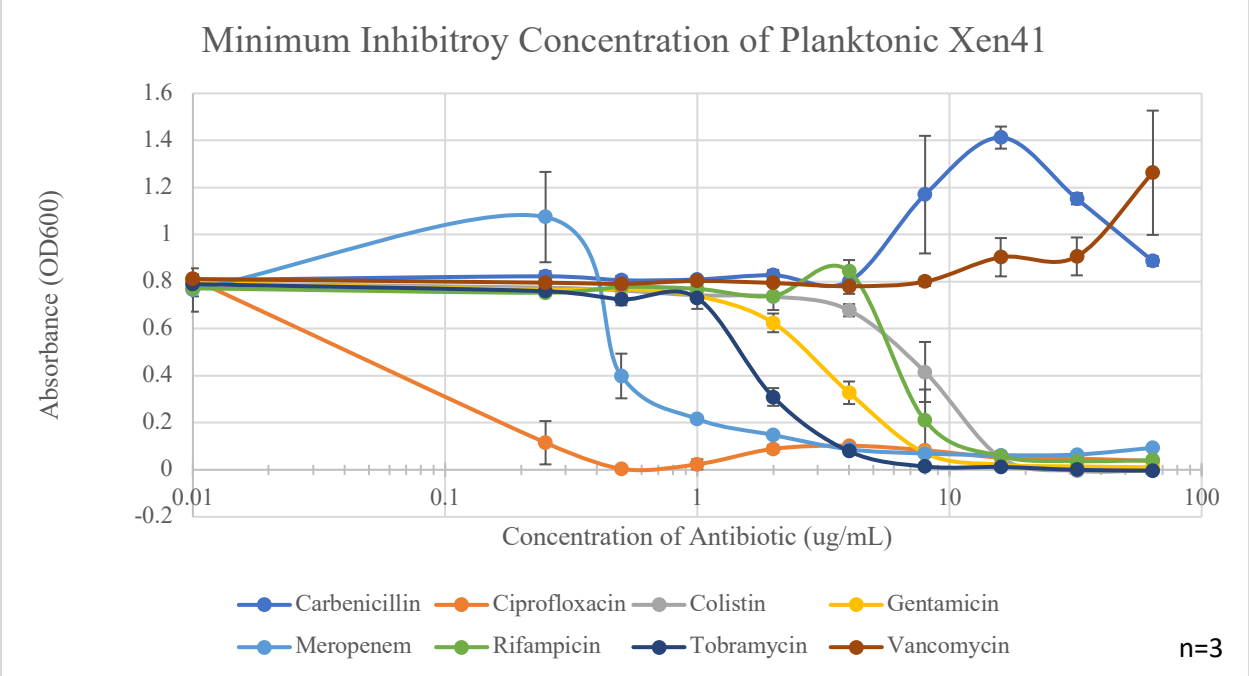
A Dunnett test was done to compare the colony density of the different treatment groups. Gentamicin was used as the control group. A Turkey test was then used to compare all the treatment group to each other. From the Turkey test, the best treatment option that lead to the fewest variant colony can be determined.

Results and Analysis

Results 1: MIC for Planktonic SAP231 and Xen41



Graph 1: The concentrations of antibiotic that inhibits the growth of SAP231. SAP231 showed resistance towards carbenicillin and colistin while being susceptible to ciprofloxacin, gentamicin, meropenem, rifampicin, tobramycin, and vancomycin.

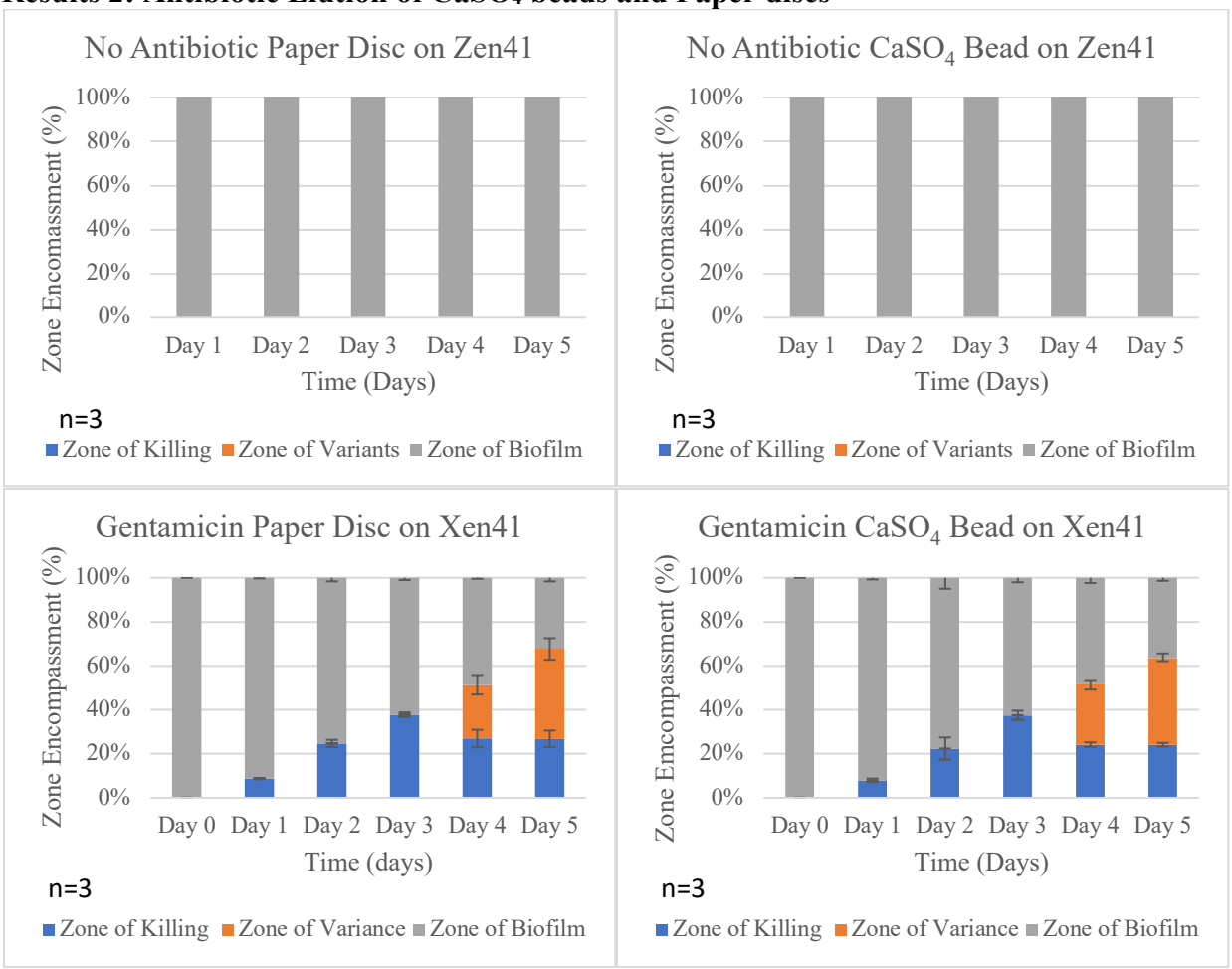


Graph 2: The concentrations of antibiotic that inhibits the growth of Xen41. Xen41 showed resistance towards carbenicillin and vancomycin, while being susceptible towards ciprofloxacin, gentamicin, meropenem, rifampicin, and tobramycin.

Table 2: MIC of Planktonic Bacteria		
Antibiotics	SAP231	Xen41
Carbenicillin	Resistant	Resistant
Ciprofloxacin	8µg/mL	0.5µg/mL
Colistin	Resistant	16µg/mL
Gentamicin	32µg/mL	8µg/mL
Meropenem	8µg/mL	4µg/mL
Rifampicin	0.25µg/mL	16µg/mL
Tobramycin	32µg/mL	4µg/mL
Vancomycin	4µg/mL	Resistant

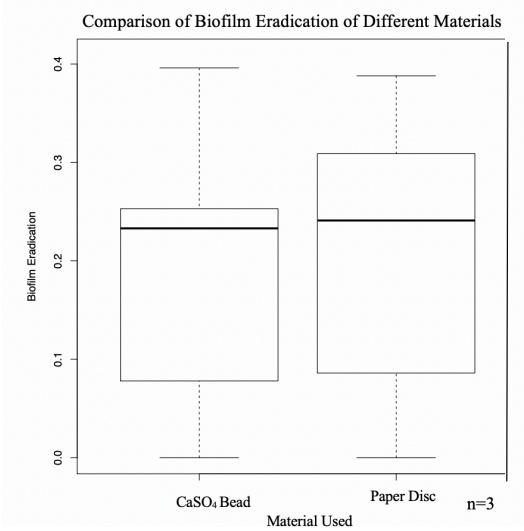
The MIC of each antibiotic was found for Xen41 and SAP231. The MICs were measure by looking at the Concentration of Antibiotic vs OD_{600} graphs. The values were determined by the bottom plateau of the graph where the OD remained stagnant, which indicates no growth. Planktonic SAP231 and Xen41 were both susceptible to six of the eight antibiotics (Table 1). Rifampicin is most potent towards SAP231, while ciprofloxacin was most potent towards Xen41. Potency is determined by the amount of drug required to produce an effect.

Results 2: Antibiotic Elution of $CaSO_4$ beads and Paper discs



Graph 3: The elution of gentamicin on Xen41 biofilm can be seen over the course of 5 days. The zone of killing gradually increase with the appearance of a variant zone on day 4. These graphs show the elution kinetics between two different antibiotics containing vessels $CaSO_4$ beads and paper discs. (As a control, the biofilm remained intact 100% all 5 days with just $CaSO_4$ bead and paper disc with no antibiotics. (Percentages based on a 78.53cm^2 biofilm.)

The elution of gentamicin can be seen on Xen41 over the course of time and with the different materials (Figure 2, Graph 3). Day 0 indicates the state of the biofilm when the antibiotic is first placed. The biofilm is completely intact on Day 0. As the antibiotic elutes outwards the zone of killing is increased. Day 4 is when variant colonies appear which is indicated by the zone of variants. It is worth noting that the zone of variants shows up within the zone of killing thus decreasing the zone of killing.



Graph 4: The box and whisker plots graphed the means of the biofilm eradication of two different antibiotic materials, $CaSO_4$ beads and paper discs. Biofilm eradication takes into account the combined areas of the zone of killing and the zone of variants. The y-axis shows the biofilm eradication while the x-axis shows the different materials. It is concluded that there is no statistical difference between the two materials with a p-value of 0.533.

To test the significance of the data between the two materials, an ANOVA test was done. This test can show that the treatment groups performed similarly or can yield statistically different results. The null hypothesis is that there is no difference between the two treatment groups. The alternative hypothesis is that there is a difference between the two treatment groups. The ANOVA test showed that the data yielded a p-value of 0.533. Using a confidence level of 0.05 with the p-value being greater than 0.05 (0.533>0.05), we fail to reject the null hypothesis concluding that there is no difference between the treatment groups.

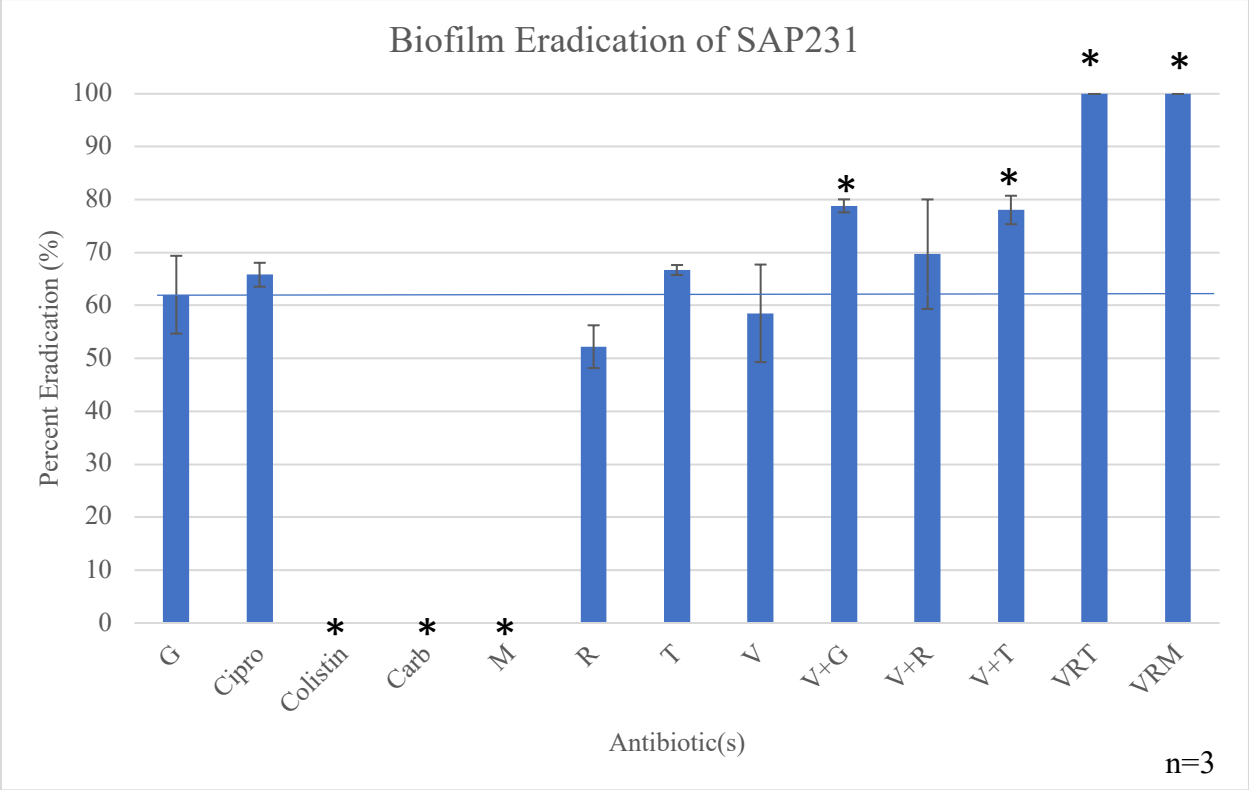
Results 3: Single and Combinatory Antibiotic(s) against Biofilm

Table 3: Preliminary Experiment: Antibiotic Elution on Agar Plate after 5 Days		
Antibiotics	**SAP231**	**Xen41**
Carbenicillin	Complete Growth	Few Colonies
Ciprofloxacin	Few Colonies	No Growth
Colistin	Complete Growth	No Growth
Gentamicin	No Growth	No Growth
Meropenem	No Growth	No Growth
Rifampicin	No Growth	Few Colonies
Tobramycin	No Growth	No Growth
Vancomycin	No Growth	Complete Growth

The preliminary experiment shows that the antibiotic is not hindered by the agar. The results show that the antibiotic was able to diffuse completely throughout the agar plate reaching MIC high enough to prevent the growth of bacteria. The actual concentration of antibiotic in the agar is unknown because it is unknown if the antibiotic in the bead completely diffused out into the media. Assuming if all the antibiotic did diffuse out the maximum concentration of antibiotic on the plate is 50µg/mL. The concentration of antibiotic on the plate is somewhere between 50µg/mL and the MIC listed in Table 2.

$$Concentration\ of\ Antibiotic = \frac{Amount\ of\ Antibiotic}{Volume\ of\ Media} = \frac{1000\mu g}{20mL} = 50\mu g/mL$$

Comparing these results to planktonic susceptibility, it is worth noting that carbenicillin is shown as resistant according to the OD growth curve. On the agar plate it appears that Xen41 is susceptible to carbenicillin. Overall, after 5 days of incubation the concentration of antibiotic on the plate should be enough to inhibit the growth of bacteria.



Graph 5: Biofilm eradication is measured by combining the zone of killing and the zone of variants. Biofilm eradication was compared to gentamicin. An asterisk (*) marks significance. An asterisk below the average of gentamicin, set by the blue line, indicates significantly less, while an asterisk above the line indicates significantly more. (Percentages based on 78.53cm^2 biofilm.)

Out of the single antibiotics, carbenicillin, colistin, and meropenem were not able to eradicate the biofilm. Comparing this to the results of planktonic eradication, meropenem was effective against planktonic bacteria, but not towards a biofilm. Carbenicillin and colistin did not have an effect on planktonic and biofilm bacteria, thus concluding that SAP231 is resistant towards these two antibiotics. The triple combination VRT and VRM were able to completely eradicate the biofilm.

Table 4: Dunnett Test- Biofilm Eradication of SAP231					
Comparison	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G vs. Carb	-0.6203	-0.7573 to -0.4833	Yes	****	<0.0001
G vs. Cipro	0.03771	-0.09927 to 0.1747	No	ns	0.9978
G vs. Colistin	-0.6203	-0.7573 to -0.4833	Yes	****	<0.0001
G vs. M	0.6203	0.4833 to 0.7573	Yes	****	<0.0001
G vs. R	0.0982	-0.03878 to 0.2352	No	ns	0.3552
G vs. T	-0.04665	-0.1836 to 0.09034	No	ns	0.9862
G vs. V	0.03515	-0.1018 to 0.1721	No	ns	0.9988
G vs. V+G	-0.1679	-0.3048 to -0.03088	Yes	**	0.0073
G vs. V+R	-0.07659	-0.2136 to 0.06039	No	ns	0.7048
G vs. V+T	-0.1601	-0.2971 to -0.02313	Yes	*	0.0121
G vs. VRT	-0.3797	-0.5167 to -0.2427	Yes	****	<0.0001
G vs. VRM	-0.3797	-0.5167 to -0.2427	Yes	****	<0.0001

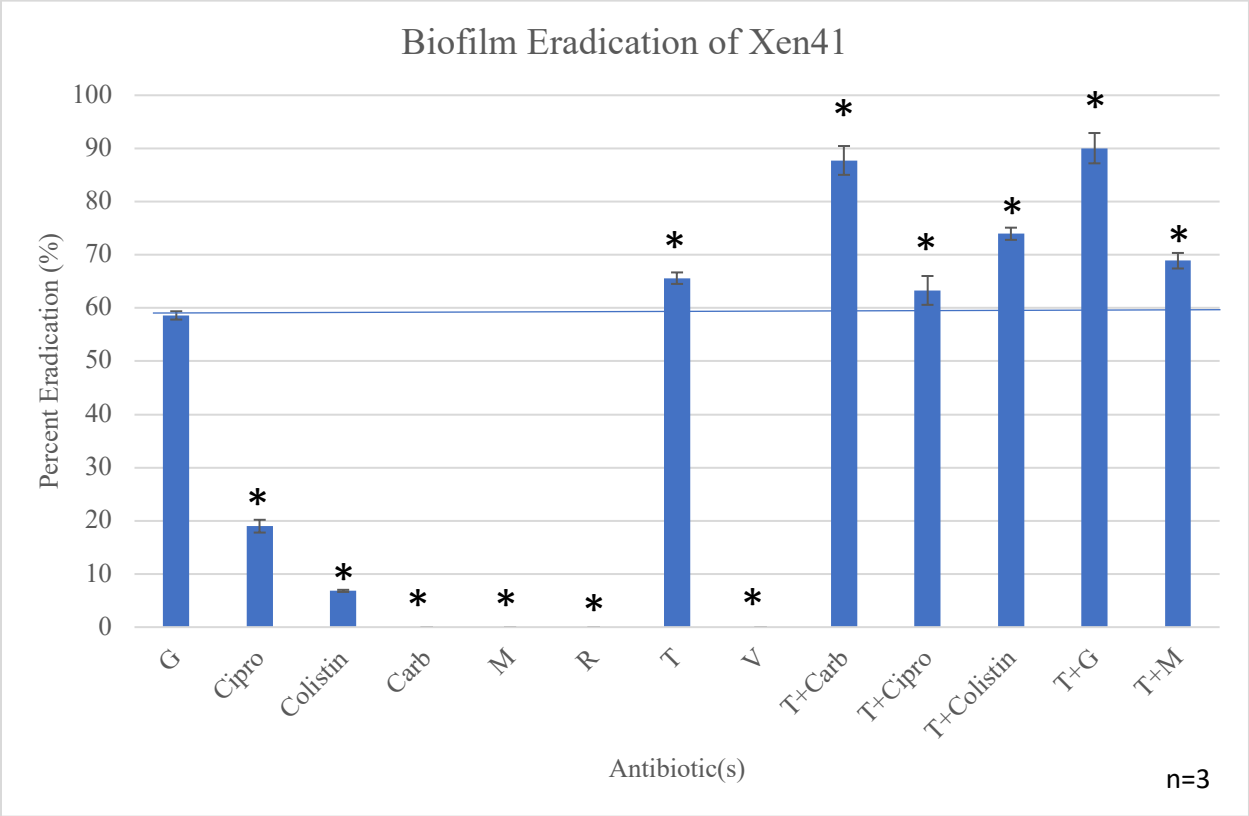
Table 4: A Dunnett test was done to compare the treatment groups towards the control, gentamicin. Alpha level was set at 0.05. Gray highlighted box indicates no significance, red highlight box indicates significantly less, and a green highlighted box indicates significantly more.

A Dunnett test was done to compare the other treatment groups towards the control, gentamicin. The treatment using ciprofloxacin, rifampicin, tobramycin, vancomycin, and V+R did not produce any significant results than gentamicin. Treatment using carbenicillin, colistin, and meropenem eradicated the biofilm significantly. Looking at the combinatory antibiotic, V+G, V+T, VRT, and VRM performed significantly better than gentamicin (Table 4).

Table 5: Turkey Test- Biofilm Eradication of SAP231					
Comparison	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
VRM vs. VRT	0	-0.137 to 0.137	No	ns	>0.9999
VRM vs. V+T	-0.2196	-0.3566 to -0.08258	Yes	***	0.0002
VRM vs. V+G	-0.2118	-0.3488 to -0.07483	Yes	***	0.0004
VRT vs. V+T	-0.2196	-0.3566 to -0.08258	Yes	***	0.0002
VRT vs. V+G	-0.2118	-0.3488 to -0.07483	Yes	***	0.0004
V+G vs. V+T	0.007748	-0.1292 to 0.1447	No	ns	>0.9999

Table 5: A Turkey test compared antibiotic treatment group to all other treatment group. Alpha level was set at 0.05. These values show only the comparisons that performed significantly better than gentamicin. Gray highlighted box indicates no significance and a green box indicates significantly more.

A Turkey test was done to find out which treatment group eradicated the biofilm the best. Out of the 78 comparisons, the treatment groups that performed the better than gentamicin are listed (Table 5). Overall the triple combination of VRT and VRM performed significantly better than the double combination of V+G and V+T. There is no significance between VRT and VRM. Thus, this concludes that combinatory antibiotics were most effective with VRT and VRM being the best at eradication the biofilm.



Graph 6: Biofilm eradication is measured by combining the zone of killing and the zone of variants. Biofilm eradication was compared to gentamicin. An asterisk (*) marks significance. An asterisk below the average of gentamicin, set by the blue line, indicates significantly less, while an asterisk above the line indicates significantly more. (Percentages based on 78.53cm^2 biofilm.)

Table 6: Dunnett Test- Biofilm Eradication of Xen41					
Dunnentt Test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G vs. Carb	-0.5861	-0.6308 to -0.5414	Yes	****	<0.0001
G vs. Cipro	-0.3963	-0.441 to -0.3515	Yes	****	<0.0001
G vs. Colistin	-0.5179	-0.5626 to -0.4732	Yes	****	<0.0001
G vs. M	0.5861	0.5414 to 0.6308	Yes	****	<0.0001
G vs. R	0.5861	0.5414 to 0.6308	Yes	****	<0.0001
G vs. T	-0.07004	-0.1148 to -0.02532	Yes	***	0.0003
G vs. V	0.5861	0.5414 to 0.6308	Yes	****	<0.0001
G vs. T+Carb	-0.2914	-0.3361 to -0.2467	Yes	****	<0.0001
G vs. T+Cipro	-0.04701	-0.0917 to -0.002298	Yes	*	0.0329
G vs. T+Colistin	-0.1535	-0.1983 to -0.1088	Yes	****	<0.0001
G vs. T+G	-0.3144	-0.3592 to -0.2697	Yes	****	<0.0001
G vs. T+M	-0.1029	-0.1476 to -0.05815	Yes	****	<0.0001

Table 6: A Dunnett test was done to compare the treatment groups towards the control, gentamicin. Alpha level was set at 0.05. Red highlight box indicates significantly less, and a green highlighted box indicates significantly more.

Out of the single antibiotics, carbenicillin, meropenem, rifampicin, and vancomycin were not able to eradicate the biofilm. Comparing this to the results of planktonic eradication, meropenem and rifampicin are effective against planktonic bacteria, but not towards a biofilm. Carbenicillin and vancomycin did not have an effect on planktonic and biofilm bacteria, thus concluding that Xen41 is resistant towards these two antibiotics. All of the double combination and tobramycin have high clearance of biofilm.

A Dunnett test was done to compare the other treatment groups towards the control, gentamicin. All of the treatment group produced significant results. Out of the single antibiotics, tobramycin performed significantly better than gentamicin with all other single antibiotics, ciprofloxacin, colistin, carbenicillin, meropenem, rifampicin, and vancomycin, performed significantly worse. All of the combinatory antibiotics, T-Carb, T-Cipro, T-Colistin, T+G, and T+M performed significantly better than gentamicin.

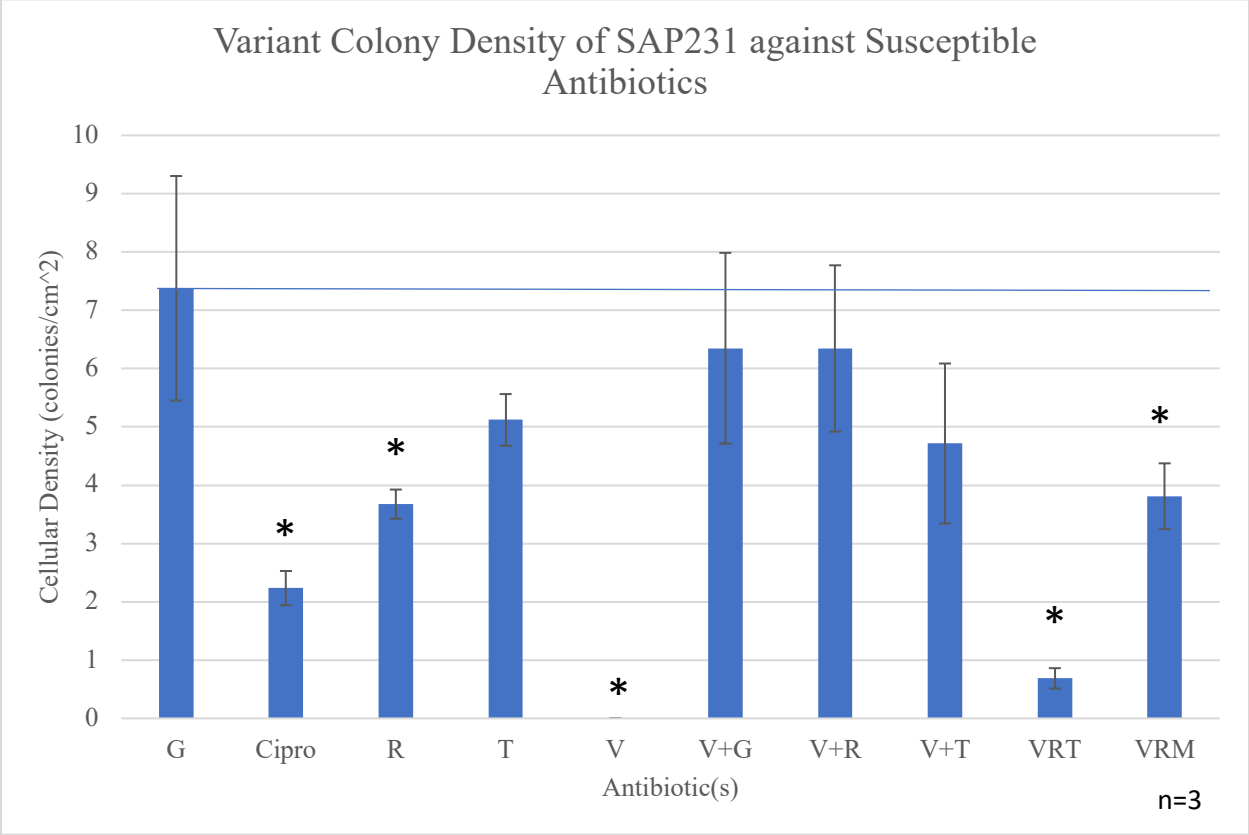
Table 7: Turkey Test- Biofilm Eradication of SAP231

Dunnentt Test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
T+Carb vs. T	-0.2214	-0.2661 to -0.1766	Yes	****	<0.0001
T+Cipro vs. T	0.02303	-0.02169 to 0.06774	No	ns	0.7966
T+Colistin vs. T	-0.0835	-0.1282 to -0.03879	Yes	****	<0.0001
T+G vs. T	-0.2444	-0.2891 to -0.1997	Yes	****	<0.0001
T+M vs. T	-0.03282	-0.07754 to 0.01189	No	ns	0.3227
T+Carb vs. T+Colisin	0.1379	0.09314 to 0.1826	Yes	****	<0.0001
T+Carb vs. T+G	-0.02306	-0.06777 to 0.02166	No	ns	0.7953
T+G vs. T+Colistin	-0.1609	-0.2056 to -0.1162	Yes	****	<0.0001

Table 5: A Turkey test compared antibiotic treatment group to all other treatment group. Alpha level was set at 0.05. These values show only the comparisons that performed significantly better than gentamicin. Gray highlighted box indicates no significance and a green box indicates significantly more.

A Turkey test was done to find out which treatment group eradicated the biofilm the best. Out of the 78 comparisons, the treatment groups that performed the better than gentamicin are listed (Table 7). First, the comparison of the other antibiotics was done against tobramycin, since tobramycin was the only single antibiotic that performed better than gentamicin. It is concluded that the double combination, T+Carb, T+Colistin, and T+G performed better than tobramycin alone, while T+Cipro and T+M were not significantly different. Out of T+Carb, T+G, and T+Colistin, T+Carb and T+G performed better than T+Colistin and were not significantly different from each other. Thus, concluding that the combination of T+Carb and T+G performed the best.

Result 4: Colony Density of Variant Colonies



Graph 7: Variant colony density was found by quantifying the number of variant colonies in the zone of variants dividing the total area of the zone. Variant colony density was compared against gentamicin. An asterisk (*) marks significance. An asterisk below the average of gentamicin, set by the blue line, indicates significantly fewer colonies.

Out of the single antibiotics, gentamicin and tobramycin are correlated with the highest number of variant colonies. Gentamicin and tobramycin are both aminoglycoside, which means there might be a correlation between variant colony appearance and aminoglycosides. Vancomycin was the only treatment option that had zero variant colonies.

Table 8: Dunnett Test- Variant Colony Density of SAP231

Comparison	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G vs Cipro	-5.143	-7.872 to -2.415	Yes	****	<0.0001
G vs. R	3.703	0.9739 to 6.431	Yes	**	0.0022
G vs. T	2.257	-0.4716 to 4.986	No	ns	0.1807
G vs. V	7.378	4.649 to 10.11	Yes	****	<0.0001
G vs. V+G	1.029	-1.7 to 3.757	No	ns	0.9699
G vs. V+R	1.032	-1.696 to 3.761	No	ns	0.9692
G vs. V+T	2.663	-0.06613 to 5.391	No	ns	0.0606
G vs. VRT	6.691	3.963 to 9.42	Yes	****	<0.0001
G vs. VRM	3.567	0.8381 to 6.295	Yes	**	0.0035

Table 8: A Dunnett test was done to compare the treatment groups towards the control, gentamicin. Alpha level was set at 0.05. Gray highlighted box indicates no significance, red highlight box indicates significantly less variant colony density.

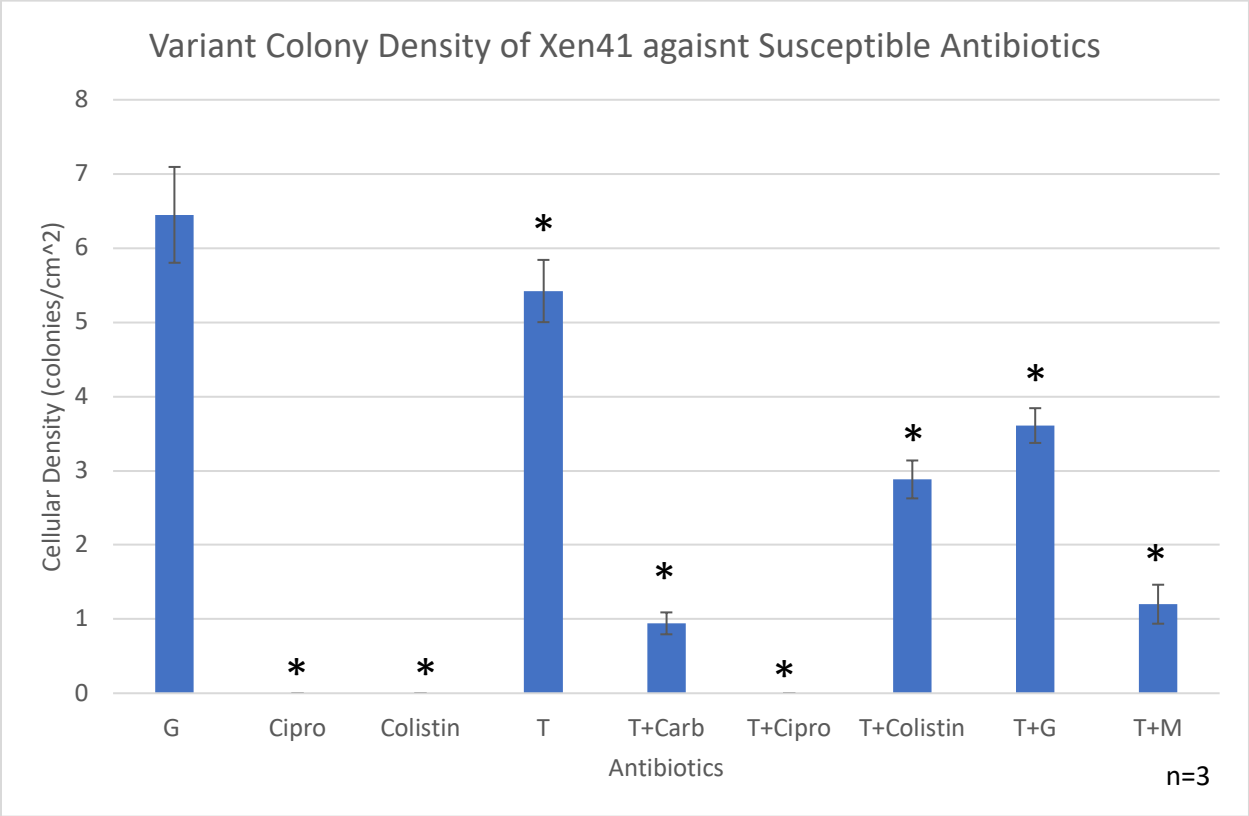
Using a Dunnett test comparing treatment groups to gentamicin (Table 8), only ciprofloxacin, rifampicin, and vancomycin had significantly fewer colonies. Fewer colonies would indicate a better treatment option. Compared to the combinatory antibiotics, it is surprising to see that the treatment with V+R, V+T, and V+G are not significantly different from gentamicin. However, the triple combination of VRT and VRM did have significantly fewer variants.

Table 9: Turkey Test- Variant Colony Density of SAP231

Comparison	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
V vs. Cipro	2.235	-0.4942 to 4.963	No	ns	0.191
V vs. R	3.675	0.9467 to 6.404	Yes	**	0.0025
V vs. VRT	-0.6867	-3.415 to 2.042	No	ns	0.999
V vs. VRM	-3.811	-6.54 to -1.083	Yes	**	0.0016
VRT vs. Cipro	1.548	-1.181 to 4.276	No	ns	0.6868
VRT vs. VRM	-3.125	-5.853 to -0.3959	Yes	*	0.0148
VRT vs. R	2.989	0.26 to 5.717	Yes	*	0.0227
Cipro vs. R	-1.441	-4.17 to 1.288	No	ns	0.7708
Cipro vs. VRM	-1.577	-4.305 to 1.152	No	ns	0.6629
Rifampicin vs. VRM	-0.1358	-2.865 to 2.593	No	ns	>0.9999

Table 9: A Turkey test compared antibiotic treatment group to all other treatment group. Alpha level was set at 0.05. These values show only the comparisons that performed significantly better than gentamicin. In this case, having fewer colonies means it is a better treatment. Gray highlighted box indicates no significance and a red box indicates significantly less.

The Turkey test compared each treatment group to each other (Table 9). The values selected for the table were the treatment groups that performed better than gentamicin. Since vancomycin had the least average of variant colonies, it was compared against all other treatment groups. The results show that ciprofloxacin and the combination VRT were not significantly different from vancomycin. This concludes that vancomycin, ciprofloxacin, and VRT were the best treatment option for reduction of variant colonies in SAP231.



Graph 8: Variant colony density was found by quantifying the number of variant colonies in the zone of variants dividing the total area of the zone. Variant colony density was compared against gentamicin. An asterisk (*) marks significance. An asterisk below the average of gentamicin, set by the blue line, indicates significantly fewer colonies.

Out of the single antibiotics, gentamicin and tobramycin are correlated with the highest number of variant colonies similarly to SAP231. Aminoglycosides had the highest variant colony appearance. Only three of the treatment groups, ciprofloxacin, colistin and T+Cipro, had zero variant colonies.

Table 10: Dunnett Test- Variant Colony Density of Xen41

Comparison	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G vs Cipro	-6.449	-7.187 to -5.711	Yes	****	<0.0001
G vs. Colistin	-6.449	-7.187 to -5.711	Yes	****	<0.0001
G vs. T	1.027	0.2886 to 1.765	Yes	**	0.0016
G vs. T+Carb	5.506	4.768 to 6.244	Yes	****	<0.0001
G vs. T+Cipro	6.449	5.711 to 7.187	Yes	****	<0.0001
G vs. T+Colistin	3.566	2.828 to 4.304	Yes	****	<0.0001
G vs. T+G	2.84	2.102 to 3.578	Yes	****	<0.0001
G vs. T+M	5.248	4.51 to 5.986	Yes	****	<0.0001

Table 10: A Dunnett test was done to compare the treatment groups towards the control, gentamicin. Alpha level was set at 0.05. Gray highlighted box indicates no significance, red highlight box indicates significantly less variant colony density.

Using a Dunnett test comparing treatment groups to gentamicin (Table 10), all of the single antibiotics, ciprofloxacin, colistin, and tobramycin had significantly fewer colonies than gentamicin. Fewer colonies would indicate a better treatment option. All of the combinatory antibiotics, T+Carb, T+Cipro, T+Colistin, T+G, and T+M had significantly fewer variants than gentamicin.

Table 11: Turkey Test- Variant Colony Density of Xen41					
Comparison	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Cipro vs. Colistin	0	-0.738 to 0.738	No	ns	>0.9999
Cipro vs. T	-5.422	-6.16 to -4.684	Yes	****	<0.0001
Cipro vs. T+Carb	-0.9423	-1.68 to -0.2043	Yes	**	0.0046
Cipro vs. T+Cipro	0	-0.738 to 0.738	No	ns	>0.9999
Cipro vs. T+Colistin	-2.882	-3.62 to -2.144	Yes	****	<0.0001
Cipro vs. T+G	-3.609	-4.347 to -2.871	Yes	****	<0.0001
Cipro vs. T+M	-1.2	-1.938 to -0.4624	Yes	***	0.0002
Colistin vs. T+Cipro	0	-0.738 to 0.738	No	ns	>0.9999

Table 11: A Turkey test compared antibiotic treatment group to all other treatment group. Alpha level was set at 0.05. These values show only the comparisons that performed significantly better than gentamicin. In this case, having fewer colonies means it is a better treatment. Gray highlighted box indicates no significance and a red box indicates significantly less.

The Turkey test compared each treatment group to each other (Table 11). Not all of the values are listed above. Since ciprofloxacin, colistin, and T+Cipro, had the same mean of 0, the mean difference and adjusted p-value are the same as the comparison of ciprofloxacin vs. all other drugs. The results show that ciprofloxacin, colistin, and T+Cipro had the fewest variant colonies and that they themselves are not significantly better to each other. This means that ciprofloxacin, colistin, and T+Cipro are the best treatment options for variant colony reduction in Xen41.

Discussion

From the data shown, biofilms are more resilient towards antibiotic treatment. Comparing the MIC values of planktonic bacteria and the susceptibility of biofilms, SAP231 and Xen41 are more susceptible when planktonic. SAP231 was susceptible towards ciprofloxacin, meropenem, gentamicin, rifampicin, tobramycin, and vancomycin when planktonic (Table 2), but when it is in a biofilm, SAP231 was not susceptible towards meropenem (Graph 5). Xen41 was susceptible towards carbenicillin (Table 3), ciprofloxacin, colistin, meropenem, gentamicin, rifampicin, and tobramycin when planktonic (Table 2), but when it is in a biofilm, Xen41 was not susceptible towards meropenem and rifampicin (Graph 7). From the results, when Xen41 was in a biofilm beta-lactam drugs like meropenem and carbenicillin were not susceptible at clearing the biofilm compared to the preliminary susceptibility test (Table 3). A similar event happened with SAP231, where meropenem was not able to kill the biofilm, while carbenicillin was resistant. Based on these observations, there might be some mechanisms of the biofilm that is inhibiting the efficacy of beta-lactam drugs.

Although bacterial biofilms are susceptible towards some of the antibiotics, large portions of the biofilm remained intact after incubation with antibiotics. None of the single combination antibiotics were able to completely eradicate the zone of biofilm of SAP231 or Xen41. It is found that the concentration of antibiotic can diffuse well above the MIC (Table 3). This could mean the biofilm is preventing the penetration and diffusion of antibiotic or the biofilm itself is becoming tolerant towards the antibiotics. It is hypothesized that the antibiotics that did not lead to variant colonies, like colistin and ciprofloxacin for Xen41 (Graph 6) and vancomycin for SAP231 (Graph 5), the antibiotic was not able to diffuse thoroughly throughout the plate because no variant colonies formed, and the biofilm was not completely eradicated. The antibiotics that lead to the appearance of variant colonies might be gaining tolerance and resistances towards the antibiotic because the variant colonies expressed those phenotypes when re-plated onto fresh agar.

Variant colonies encompass many phenotypes from tolerant to resistant. It appears that there is some relationship between aminoglycosides, like tobramycin and gentamicin, and the appearance of variant colonies. Both antibiotics had the highest variant cell density of any of antibiotic in SAP231 and Xen41 (Graph 7 and 9). Looking at SAP231, the variants of rifampicin and ciprofloxacin are probably an induced genetic mutation that allows the bacteria to gain resistance since the colony density was much lower than the aminoglycosides like gentamicin and tobramycin. It is found that a single base pair change in the β sub-unit of RNA polymerase confers with high levels of rifampicin resistance.[15] Similarly with ciprofloxacin resistance, an amino acid substitution can decrease the binding affinity of ciprofloxacin to DNA gyrase or topoisomerase IV.[16] Ciprofloxacin and rifampicin have the highest area of killing (Graph 5) with the lowest variant colony density (Graph 9). This would also explain why the MIC elevated to resistant after 5 days of incubation (Table 2).

Summary Table: Antibiotic Efficacy

Ranking	Planktonic SAP231 (Potency)	Planktonic Xen41 (Potency)	Biofilm Eradication Sap231	Biofilm Eradication Xen41	Variant Colony Reduction SAP231	Variant Colony Reduction Xen41
1	Rifampicin (0.25ug/mL)	Ciprofloxacin (0.5ug/mL)	Vancomycin+ Rifampicin+ Meropenem (100%)	Tobramycin+ Gentamicin (90.0%)	Vancomycin (0 CFU/cm^2)	Tobramycin+ Ciprofloxacin (0 CFU/cm^2)
2	Vancomycin (4ug/mL)	Tobramycin (4ug/mL)	Vancomycin+ Rifampicin+ Tobramycin (100%)	Tobramycin+ Carbenicillin (87.5%)	Vancomycin+ Rifampicin+ Tobramycin (0.68 CFU/cm^2)	Ciprofloxacin (0 CFU/cm^2)
3	Meropenem (8ug/mL)	Meropenem (4ug/mL)	Vancomycin+ Gentamicin (78.8%)	Tobramycin+ Colistin (73.9%)	Ciprofloxacin (2.23 CFU/cm^2	Colistin (0 CFU/cm^2)

The summary table highlights the important findings in this study. Antibiotic treatment options are ranked according to their efficacy. For planktonic bacteria, the rankings were done according to potency with rifampicin being the best for SAP231 and ciprofloxacin for Xen41. A lot of the

antibiotics are effective in treating planktonic bacteria. When it comes to biofilms, antibiotic efficacy was ranked according to biofilm clearance. Over all combinatory antibiotics worked best with triple combination of VRT and VRM being effective in treating SAP231 and double combination of T+G and T+Carb being effective in treating Xen41. Variant colonies were ranked according to which antibiotic had the fewest colonies with vancomycin working best towards SAP231 with zero colony appearance. T+Cipro, ciprofloxacin, and colistin worked best with zero variant colony appearance. It is important to note there was not a single combination that worked best at clearing the biofilm and had the fewest variant colonies. It is important to weigh the risk and benefits of the different treatment options. Some instances using the combination of antibiotic that is able to clear the biofilm is better because it does reduce the number of bacteria in the host and in this case the host immune system can fight against the variant bacteria. Another instance if the host immune system is compromised choosing a combination that had the fewest variant colonies may be better because the host immune system may not be strong enough to fight variants. Looking at the summary table based on the number of times a certain antibiotic appeared, vancomycin is very effective against *S. aureus*, while ciprofloxacin is very effective against *P. aeruginosa*.

Conclusion

Differentiating if an infection is a biofilm is important because it does significantly change how physicians should approach the infection. Treating biofilms are more difficult than treating planktonic infections, as seen in this *in vitro* study. None of the antibiotics were able to completely eradicate the biofilm, but were effective at killing or preventing growth in planktonic cultures. When compared to antibiotic susceptibility of planktonic vs biofilm, some antibiotics may not be effective at treating biofilms although they are effective when planktonic. The appearance of variant colonies with the use of antibiotics is concerning because it shows that antibiotic treatment is not as effective at killing the pathogen as thought. The antibiotics may look like it is susceptible and effective for the first couple days, but as time progresses, it can give rise to these variant bacteria. Treating variant bacteria may be more difficult than treating the original pathogen as these variants are able to survive the effects of the antibiotic. It is unsure if these variant colonies have an impact *in vivo*, but looking at the *in vitro* study, variants are able to survive high concentration of antibiotic well above the MIC compared to planktonic bacteria. Accounting for variants, it is important to use the most effective antibiotic treatment that has the greatest efficacy towards the pathogen without inducing the growth of variants. Using a combination of antibiotics with multiple different mechanism of action can best clear a biofilm. It is also important to have many effective combinations of antibiotics to slow the development of antibiotic resistance.

Future Work

The future of this project is to investigate if the variant phenomenon occurs *in vivo*, using a wax worm model (Project led by Devin Sindeldecker). Currently, *P. aeruginosa* mutants are being screened to look to see if there is a transcriptional change that is related to the appearance of variant colonies. The idea is to translate the findings of antibiotics combinations to these mutants to see if the results and findings are similar. *In vitro* tests on clinical isolates have shown that this variant phenomenon is occurring. Some clinical isolates that have been tested do not show the same susceptibility pattern as Xen41. Further antibiotic combination testing will be looked at, introducing new classes of drugs like cephalosporins, testing for biofilm eradication.

Appendix

Different Zones Encompassment on SAP231 Biofilm Day 9				
Rank	**Antibiotics**	**Zone of Killing**	**Zone of Variants**	**Zone of Biofilm**
1	VRM	58.5%	41.4%	0%
1	V	58.5%	0%	41.4%
2	VRT	57.6%	42.3%	0%
3	V+T	52.3%	25.7%	21.9%
4	V+G	52.1%	26.6%	21.8%
5	T	44.4%	22.2%	33.3%
6	V+R	40.1%	29.5%	30.3%
7	G	37.5%	26.2%	37.9%
8	Cipro	2.15%	63.6%	34.1%
9	R	2.08%	50.1%	47.7%
10	Carb	Not Susceptible 100% Biofilm		
10	Colistin	Not Susceptible 100% Biofilm		
10	M	Not Susceptible 100% Biofilm		

This tables shows the zone percent encompassment of each zone in SAP231 biofilm. Biofilm eradication included both the zone of killing and the zone of variants. (Percentages based on 78.53cm^2 biofilm.)

Different Zones Encompassment on Xen41 Biofilm Day 5				
Rank	**Antibiotics**	**Zone of Killing**	**Zone of Variants**	**Zone of Biofilm**
1	T+Cipro	58.5%	0%	36.6%
2	T+M	42.1%	26.7%	31.1%
3	T+G	34.5%	55.4%	9.94%
4	T	28.0%	37.5%	34.3%
5	T+Colistin	25.4%	48.4%	48.4%
6	T+Carb	20.8%	66.8%	66.8%
7	Cipro	18.9%	0%	81.0%
8	G	12.5%	46%	43.1%
9	Colistin	6.8%	0%	93.1%
10	Carb	Not Susceptible 100% Biofilm		
10	M	Not Susceptible 100% Biofilm		
10	R	Not Susceptible 100% Biofilm		
10	V	Not Susceptible 100% Biofilm		

This tables shows the zone percent encompassment of each zone in Xen41 biofilm. Biofilm eradication included both the zone of killing and the zone of variants. (Percentages based on 78.53cm^2 biofilm.)

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